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Isolation and Identification of Plant Growth-Promoting Rhizobacteria (PGPR) from the Rhizosphere of Sugarcane in Saline and Non-Saline Soil

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ABSTRACT

Soil salinity is one of the limiting factors of agricultural production in arid and semi-arid areas that reduces yields and optimal crop production. Awareness of rhizosphere bacterial diversity and use of salinity-resistant bacteria is considered as a critical strategy to increase plant growth in these areas. This study aimed to determine the population diversity of sugarcane rhizosphere bacteria in saline and non-saline soil and survey some growth-promoting properties. For this purpose, random sampling from the rhizosphere of sugarcane was performed. Bacteria were isolated by culturing serial on nutrient agar medium and were identified based on biochemical assays. The ability of isolates to fix nitrogen, dissolve phosphate and potassium and auxin production was investigated. Finally, the best growth-promoting isolates were identified based on 16S rRNA sequences. Generally, 40 bacteria were isolated from saline and non-saline soil that these strains were mainly from *Bacillus*, *Paenibacillus* and *Pseudomonas*. Salinity had the highest effect on bacterial community structure with the higher diversity of microorganisms in saline soils. Four strains were selected as growth-promoting strains which based on biochemical and phylogenetic analysis were identified as *Enterobacter cloacae* R13, *Enterobacter cloacae* R33, *Paenibacillus lactis* and *Pseudomonas* sp.

Keywords

Phosphate, Inceptisol, Potassium, Nitrogen, Auxin, *Enterobacter cloacae*.

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Introduction

Salinity challenge is one of the major problems that reduce productivity of fertile lands. Salinity affects not only plant production but also the biodiversity (Fernandez *et al.*, 2010). High levels of salinity (more than 4 dS/m) are created usually due to salts in irrigation water and fertilizers, low rainfall, high temperature, inappropriate management practices and long-term drop irrigation (Yao *et al.*,

2010). In Iran over 32 million hectares of soils are affected by salinity that covers nearly 30 percent of the entire country (34 million hectares) and 55% of arable lands (Paziraand Homae, 2010). Sugarcane culture in Khuzestan has a significant role in providing sugar. Nowadays, this industry developed in the region and produces more than half of the country's sugar. Salinity of soil under sugarcane cultivation due

irrigation practices and application of chemical fertilizer is now being a serious issue. Some of the microorganisms, particularly valuable bacteria can develop plant performance under stress condition and, improve yield (Evelin *et al.*, 2010; Sahoo and Dhal, 2009). Their abundance and activities are controlled by various physical and chemical factors. Measuring biodiversity of microbial communities for the immediate application and fundamental understanding of microbial communities are important. Plant growth promoting rhizobacteria (PGPR) employed to promote plant growth by supplying nutrients such as nitrogen through biological nitrogen fixation, phosphate and potassium through solubilization of their insoluble forms, induce phytohormones production and plant resistance to microbial pathogens and siderophore production aiding plant nutrition by chelation (Ogboand Okonkwo, 2010). Our knowledge about microbial diversity and microorganisms' activity in the rhizosphere of sugarcane is very important in understanding the plants function and this knowledge is necessary to adjust the effect of management and conservation strategies. There is no information about associated bacteria with sugarcane growing in the fields of Iran, therefore this study was conducted to isolate and characterize potential beneficial bacteria that may be present in rhizosphere of sugarcane in saline and non-saline soil.

Materials and Methods

In order to isolate rhizobacteria from the rhizosphere of sugarcane, different points of the sugarcane farm with salinity levels of around 1.5-5(dS/m) located in the agro-industrial region of Khuzestan DebalKhazaei (42 11''31°05 ' North and 43.1'' 48° 30' East) were sampled in October 2014. Rhizosphere soil was collected by sampling the soil that was

adhered to the roots. Soil properties such as soil texture parameters (Hydrometry), pH in saturated mud, the electrical conductivity of saturated extract (ECe), Soil organic matter (the K₂Cr₂O₇-H₂SO₄ oxidation–reduction titration method), soil respiration (Anderson, 1982) and microbial biomass carbon by fumigation-extraction method were measured (Jenkinson and Powlson, 1976).

To isolate bacteria, 10g of soil was serially diluted to 10⁻⁸. About 50µl of 10⁻³ to 10⁻⁸ dilutions were spread on nutrient agar medium and incubated at 28°C for 72 h. The appeared colonies on the medium were purified based on appearance and color. Purified bacteria were examined by morphological characteristics such as shape, margin, color and pigment occurrence, elevation, texture and size of colony, after that, the bacteria were identified by standard biochemical tests on the basis of the Bergey's manual of bacterial classification (Harrigan and McCance, 1976). Microbial diversity was assayed by Shannon index (H'). The higher Shannon index reflects the higher diversity (Swingland, 2001).

$$\text{Shannon's index} \Rightarrow H = - \sum_{i=1}^R P_i * \ln P_i , \\ P_i = \frac{\text{Sample}}{\text{SUM}}$$

$$\text{Evenness index} \Rightarrow \text{Evenness} = H/H_{\max} (H_{\max} = \ln N)$$

All bacterial isolates were screened for nitrogen fixation, indole acetic acid production, and mineral phosphate and potassium solubilization by using following assays. To determine the phosphate solubility in solid medium, strains were cultured in Pikovskaya's agar (PVK) medium containing: 1% glucose, 0.5% Ca₃(PO₄)₂, 0.05% (NH₄)₂SO₄, 0.05% yeast extract, 0.01% MgSO₄.7H₂O, 0.02% NaCl, 0.00002% FeSO₄, 0.0002% MnSO₄, 0.02%

KCl and 2% Agar and set to pH 6.8-7. From overnight culture of isolates, 10µl were cultured on PVK medium in plates and incubated for 7 days at 30°C. Appearance of clear zone around the colony was regarded as their ability of the phosphate solubility. The phosphorus solubility index (SI) was calculated from the ratio of colony to halo zone diameter. The solubility of phosphorus in liquid medium was also determined in Pikovskaya's broth medium using the standard curve and determining the absorption intensity at 880 nm (Ramani, 2011). Nitrogen-fixation ability investigated according to Döbereiner (Döbereiner, 1972) and the dissolution of potassium was also performed on Aleksandrov agar medium containing vermiculite powder (Aleksandrov *et al.*, 1967). Auxin production was discovered according to Bric *et al.*, (Bric *et al.*, 1991).

Molecular identification of most suitable strains was performed through 16S rRNA sequencing. DNA was extracted using CinnaGen kit and qualified by electrophoresis on 1% agarose gel. Amplification of extracted DNA was done using general 16SrRNA primers.

The primers were forward FD1 (5'CCGAATTCGTCGACAACAGAGTTT GATCCTGGCTCAG3') and reverse RP1 (5'CCCGGGATCCAAGCTTACGGTTAC CTTGTTACGACTT3') primers (Weisburg *et al.*, 1991). Amplification cycle was included an initial denaturation (94°C, 5 min), 30 cycles each consisted of 1 min at 94°C denaturation, 40 s at 58°C annealing and 150 s at 72°C forelongation, followed by a final extension of 10 min at 72°C. Target amplification was confirmed by electrophoresis in 1% agarose containing DNA safe stain. The PCR products were sequenced and compared with the 16S rRNA sequences present in Genbank of NCBI.

Results and Discussion

Some chemical and physical properties of the soil are presented in Table 1. The soil texture of both samples with EC= 1.5 and 4.7 dS/m was clay loam and soil pH for saline and non-saline soils were 9.02 and 8.86, respectively. Microbial biomass carbon in non-saline soil was higher than saline soil. Decreased microbial biomass carbon by increasing soil salinity has been reported (Yuan *et al.*, 2007). The low respiration rate in saline soil is in agreement with other studies (Yuan *et al.*, 2007; Wichern *et al.*, 2006; Rietz and Haynes, 2003) and can be explained salt induced low osmotic potential which reduces water availability to microbes and may draw water out of the cells.

Isolation and Identification of Bacteria

Table 2 indicates the list of identified bacteria from sugarcane rhizosphere of saline and non-saline soils. Overall, 40 strains were isolated from two soil types (19 isolates from saline soil and 21 isolates from non-saline soil). According to the results presented in table 2, among 40 strains, 20 strains belonged to *Bacillus*, including 12 and 8 isolates from non-saline and saline soil, respectively. Also 9 isolates of *Paenibacillus* genus (including 6 isolates from non-saline soils and 3 ones from saline soils), 4 isolates of *Pseudomonas* (including 2 and 2 isolates isolated from non-saline and saline soil), 3 isolates of *Enterobacter* from saline soil, 1 isolate of *Micrococcus* from saline soils, 1 isolates of *Corynebacterium* from saline soils, 1 isolate of *Pediococcus* from saline soil and 1 isolate of *Arthrobacter* from non-saline soil have been diagnosed. Amongst all the bacterial isolates genera *Bacillus* was found to be the most dominant in both saline and non-saline soils followed by *Paenibacillus* in non-saline soil

and by *Paenibacillus* and *Enterobacter* in saline soil. Numbers of bacterial species associated with sugarcane rhizosphere have been isolated which belonging to *Azospirillum*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium*, and *Serratia* (Nakade Dhanraj, 2013). Diversity and evenness of isolated bacteria in both saline and non-saline soils was calculated using Shannon's index. Diversity (Shannon's Index) and evenness of isolated bacteria from saline soil was as $H=1.63$ and $E=0.840$, respectively, and these was $H=1.04$ and $E=0.753$ for non-saline soil. Diversity and evenness of isolated bacteria from sugarcane rhizosphere in saline soil were higher than non-saline soil that indicates high severity of salt stress increases diversity in the bacterial communities (Table3). This might be attributed to proliferation of halophylic bacteria in soil. Yang *et al.*, (Yang *et al.*, 2016)determined higher diversity of bacteria in the rhizosphere soil at high salinity than low. In this study, *Enterobacter*, *Corynebacterium*, *Micrococcus* and *Pediococcus* were only found in saline soil. *Pediococci* commonly grow with plant associated lactic acid bacteria in various types of forage crops. *Pediococci* have potential of fermenting xylose and arabinose, they can be used as good candidates for efficient lignocellulosic feedstock bioconversions (Boguta *et al.*, 2014). In industry, *Corynebacterium* species are used for economic production of glutamic acid (Hermann, 2003).

The production of amino acids by rhizobacteria may play an important role in the growth of plants and might also have a direct application in agricultural technologies. More research is required to determine whether those bacteria have

specific roles in the rhizosphere saline soil to induce plant toleration against salinity.

Growth Promoting Properties of Isolates

Generally, 87.5% of the isolates were able to grow in Deubernier medium and because of nitrogen fixation, color change of medium from dark green to yellow was happened (Table 4). Of these, 45.9 percent of isolates were from non-saline soil and 41.5% from saline soil. Emtiazi *et al.*, (2007) identified *Paenibacillus* as nitrogen-fixing bacterium. The different genuses of *Paenibacillus* have nitrogen fixation ability (Xie and Du, 2014).

Various reports have shown *Pseudomonas* as diazotrophic bacteria in the rhizosphere of different plants such as rice (Mirza *et al.*, 2006), sugarcane (Ramesh kumar *et al.*, 2012; Ashraf *et al.*, 2011) and legumes (Ahmad *et al.*, 2008). Khan *et al.* (2008) isolated and identified *Bacillus* and *Enterobacter* as nitrogen-fixing bacteria from the rhizosphere of rice in Bangladesh. Nitrogen-fixing enterobacteria have been isolated from sugarcane plants cultivated in other countries (Loiret *et al.*, 2004; Magnani *et al.*, 2010; Mehnaz *et al.*, 2010; Mirza *et al.*, 2001; Taulé *et al.*, 2010).

Some isolates with following codes of E1-1 (10), E1-2 (10) and E1-3 (10) isolated from saline soil and E1-9 (12) isolated from non-saline soil had the ability to produce auxin, which first 3 isolates were related to *Enterobacter* and the other was *Paenibacillus*. Change in the color intensity due to auxin production in the presence of (10) E1-1 and (10) E1-3, both were isolated from saline soil, was greater than other isolates. Earlier research reported that auxin producing *Enterobacter* sp. strain 35 isolated from sugarcane successfully colonized and promoted growth of *Brassica oleracea* (Zakria *et al.*, 2008). The

production of auxin and various other indolic and phenolic compounds by *Paenibacillus*. *Polymyxa* RP, RS and NRS isolates has been reported (Lebuhn *et al.*, 1997).

Potassium is one of the essential nutrients for plant growth and development. Most of the potassium in soil exists in various insoluble minerals (Goldstein, 1994). Microorganisms play an important role to release potassium from minerals and supply soluble K for plant. Test results of potassium dissolution by our isolates have been provided in Table 5. Isolates 10(E1-2) from saline soil had higher ratio of halo to colony diameter.

Most of the KSB obtained from the plant rhizosphere are *Bacillus* sp. and *Pseudomonas* sp (Archana *et al.*, 2013; Gopal *et al.*, 2005; Liu *et al.*, 2001; Sugumaran and Janarthanam, 2007; Zhou *et al.*, 2006). Zhang and Kong (Zhang and Kong, 2014) found 2 strains belonged to *Enterobacter cloacae* as potassium solubilizing from mica. Potassium solubilizing bacteria have been isolated from rice crops, corn crops, and coconut tree (Gopal *et al.*, 2005), tobacco (Zhang and Kong, 2014). However, there are relatively few studies on potassium solubilizing bacteria in the sugarcane rhizosphere. Ghevariya and Deasi (2014) show *Pseudomonas* sp. of potassium solubilization from mica.

The results of phosphate dissolution by isolates have been provided in Table 6. Based on these results, *Pseudomonas* (E1-2-2) with high ratio of halo to colony diameter (2.4) showed the greatest ability of insoluble phosphate dissolution in solid medium. Then *Enterobacter cloacae* [10(E1-2)] with the

ratio of halo to colony diameter (0.8) was in second place and *Bacillus* [E1-2] with a halo diameter ratio (0.75) in third place. According to the study of Mehnazet *al.*, (2010) on isolation of sugarcane rhizosphere bacteria in Punjab, 32 isolates, including *Enterobacter*, *Klebsiella* and *Pseudomonas* were detected that, 17 isolates had phosphate dissolution ability. Phosphate solubilizing microorganisms by production of phosphatase enzymes and organic acids, increase plants availability to the soluble phosphorus (Khan *et al.*, 2009).

The quantity of phosphate solubilizing by isolates in liquid medium have been provided in Table 7. Based on these results, the highest phosphate solubilization ability was respectively belong to *Bacillus* [12(K2)] from non-saline soil, *Pseudomonas*[E1-2-2] isolated from non-saline soil and *Bacillus* [2.6(K6)] from saline soil. Pearson correlation coefficient between phosphate dissolution and pH of the medium (-0.73) showed a significant negative correlation ($p < 0.01$) between these two parameters. Since the phosphate dissolution reaction occurs in rhizosphere so the probability of finding solubilizing bacteria in this area is higher (Mehta and Nautiyal, 2001). The pH reduction of the medium suggests the release of organic acids by the P-solubilizing microorganisms (Nautiyal *et al.*, 2001, Rashid *et al.*, 2004).

Figure 1 shows PCR amplification product of extracted DNA from selected bacteria in terms of growth-promoting properties, 1,500 bp product represents correct genome amplification. Results of the sequencing of the amplified fragment of each bacterium were edited using Bio edit software and were identified by Blast.

Table.1 Some physicochemical properties of soil

Properties	Saline soil	Non saline soil
Soil texture	Clay loam	Clay loam
Ec(dS/m)	4.7	1.5
pH	9.02	8.9
Organic carbon(%)	0.24	0.61
Calcium (meq/lit)	16.8	12.8
Magnesium (meq/lit)	10.9	7.36
Nitrate(meq/lit)	33.0	26.3
Bicarbonate (meq/lit)	5	4.7
Chlorine (meq/lit)	76.6	79.3
Microbial biomass carbon (mg/100gr)	324.21	477.96
Soil respiration (mgC-Co ₂ /100gsoil.1day)	4.5	4.8

Table.2 List of identified bacteria isolates

Non- saline soil		Saline soil	
Isolate code	Name of bacterial isolate	Isolate code	Name of bacterial isolate
(12)E1-4	<i>Paenibacillus</i>	(10)E1-9	<i>Paenibacillus</i>
E1-2(12)	<i>bacillus</i>	(10)E1-8	<i>Bacillus</i>
(12)E1-1	<i>bacillus</i>	(10)E1-6	<i>Pseudomonas</i>
(12)10 ⁻⁸	<i>bacillus</i>	(10)E1-5	<i>Micrococcus</i>
(1)S7	<i>bacillus</i>	(10)E1-4	<i>Bacillus</i>
(1)S2	<i>bacillus</i>	(10)E1-3	<i>Enterobacter</i>
(1)H7E	<i>bacillus</i>	(10)E1-2	<i>Enterobacter</i>
(1)G3E	<i>Paenibacillus</i>	(10)E1-1	<i>Enterobacter</i>
(1)D9E	<i>bacillus</i>	(2.6)K6	<i>Bacillus</i>
(1)A1E	<i>Arthrobacter</i>	(2.6)7-2	<i>Bacillus</i>
E1-2	<i>Bacillus</i>	K6	<i>Bacillus</i>
E1-2-2	<i>Pseudomonas</i>	K2	<i>Pseudomonas</i>
(12)K5	<i>Bacillus</i>	E1-8	<i>Paenibacillus</i>
(12)K2-1	<i>Bacillus</i>	(10)K8	<i>Paenibacillus</i>
(12)K2	<i>Bacillus</i>	(10)E3-2	<i>Bacillus</i>
(12)K1	<i>Bacillus</i>	(10)E2-4	<i>Bacillus</i>
(12)E1-10	<i>Pseudomonas</i>	(10)E2-3	<i>Bacillus</i>
(12)E1-9	<i>Paenibacillus</i>	(10)E2-2	<i>Pediococcus</i>
(12)E1-8	<i>Paenibacillus</i>	(10)E1-10	<i>Corynebacterium</i>
(12)E1-6	<i>Paenibacillus</i>		
K8	<i>Paenibacillus</i>		

Table.3 Number and shanon diversity components of isolates

	Number		Pi		ln Pi* Pi	
	Saline soil	non-Saline soil	Saline soil	non-Saline soil	Saline soil	non-Saline soil
<i>Paenibacillus</i>	3	6	0.157	0.285	-0.290	-0.356
<i>Bacillus</i>	8	12	0.421	0.571	-0.364	-0.319
<i>Pseudomonas</i>	2	2	0.105	0.095	-0.236	-0.223
<i>Enterobacter</i>	3	-	0.157	-	-0.290	-
<i>Pediococcus</i>	1	-	0.052	-	-0.153	-
<i>Corynebacterium</i>	1	-	0.052	-	-0.153	-
<i>Micrococcus</i>	1	-	0.052	-	-0.153	-
<i>Arthrobacter</i>	-	1	-	0.047	-	-0.143

Table.4 Results of nitrogen fixation ability of isolates

Saline soil		Non saline soil	
Isolate code	Nitrogen fixation	Isolate code	Nitrogen fixation
10(E1-2)	+	12(K2-1)	+
10(E1-9)	-	12(E1-8)	+
10(E1-5)	+	1(S7)	+
10(E2-4)	-	12(E1-9)	+
10(E1-4)	+	12(K5)	-
10(E2-2)	+	E1-2	+
10(E1-1)	+	12(E1-1)	+
10(E1-10)	+	K8	+
K6	+	1(S2)	+
10(E1-8)	+	1(a1)E	-
10(E1-6)	+	12(K2)	+
10(E2-3)	+	12(E1-6)	+
10(E3-2)	+	12(K1)	+
2.6(K6)	+	1(d9)E	+
10(K8)	+	12(E1-4)	+
E1-8	+	12(E1-10)	+
10(E1-3)	+	12(E1-2)	+
2.6(7-2)	+	12(10-8)	-
K2	+	E1-2-2	+
		1(h7)	+
		1(g3)	+

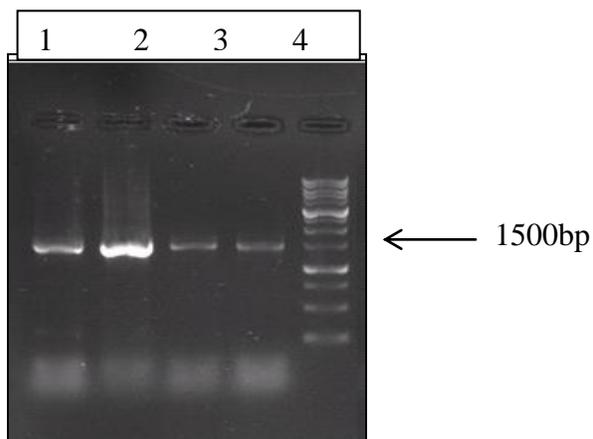
Table.5 Halo and colony diameter (mm) of isolates solubilizing potassium in Aleksandrovagar medium

Bacteria code	K ₂ HPO ₄			Vermiculite		
	Halo	Colony	Halo/Colony	Halo	Colony	Halo/Colony
10(E1-2)	7	3	2.3	-	-	-
E1-2-2	7	5	1.4	10	5	2
10(E1-3)	-	-	-	9	5	1.8
12(K1)	3	5	0.6	-	-	-

Table.6 Halo and colony diameter (mm) of isolates solubilizing phosphate in PVK agar medium and pH and quantity of phosphate solubilizing in broth PVK medium

Bacteria code	Halo	Colony	Halo/Colony	Phosphate solubilizing(mg/L)	pH
10(E1-2)	8	10	0.8	10.10	5.37
10(E1-5)	2	10	0.2	7.07	5.06
10(E1-4)	4	10	0.4	10.73	4.55
E1-2-2	12	5	2.4	13.64	4.1
10(E1-1)	4	8	0.5	10.42	5.41
1(S7)	4	10	0.4	7.06	4.84
10(E2-3)	2	8	0.25	2.95	5.26
10(E3-2)	4	10	0.4	10.42	4.82
12(E1-9)	4	8	0.5	6.68	5.7
2.6(K6)	2	10	0.2	13.53	4.8
E1-2	6	8	0.75	4.89	5.45
1(a1)E	2	10	0.2	11.19	4.48
10(E1-3)	8	10	0.8	12.13	4.2
12(K2)	6	8	0.75	14.15	4.7
K2	3	7	0.43	12.59	4.42
1(d9)E	5	9	0.55	7.77	4.84

Fig.1 PCR products of 16S rDNA from Bacteria (1500 bp).



1: 10(E1-1); 2: 12(E1-9); 3: 10(E1-3); 4: E1-2-2; 5: molecular weight marker 1 KB (Fermentas, UK)

According to the results of comparing the sequences of 16S rRNA, growth-promoting strains had 98 percent similarity with *Enterobacter cloacae* R13 [10(E1-1)], *Paenibacillus lactis* [12(E1-9)], and *Pseudomonas sp* [E1-2-2], *Enterobacter cloacae* R33 [10(E1-3)], which were deposited in Gene Bank under accession No: KX262855, KX262856, KX262854, KX262856, respectively.

In conclusion, during recent years, a great attention has been paid to saline soils due to the reducing arable land, and of the increasing demand for agricultural production of areas influenced by secondary salinization processes. Actually salt-affected soils may have a biotechnological potential in their microbial communities, which represent a reserve for future exploitation in biotechnological applications. A survey of available literature, suggests that

microbiology of saline soil and exploitation of microorganisms from these soil has not been dealt extensively. As mentioned in the introduction, very few studies have considered in addressing the diversity of PGPR, in relation to salinity. According to our results salinity had the strongest effect on bacterial community structure. On completing this investigation, I am impressed with the wide diversity of microorganisms present in saline soils. Our study revealed a high plasticity of bacterial phyla that evidently possess genera and species adaptable to salinity conditions with plant growth promoting properties. There is scope for use of nitrogen fixer and phosphate and potassium solubilizing bacteria as potential biofertilizers for reclamation saline soils of local area because isolates belongs to the same soil. From 40 isolates on two saline and non- saline soils 4 isolates having good plant growth promotion were chosen and will be suggested to produce for sugarcane cultivation in the future.

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